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Purification and Characterization of the Periplasmic Domain of the Aspartate Chemoreceptor*

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In order to facilitate biochemical studies of cell-surface receptors, a plasmid allowing the expression of the periplasmic domain of the aspartate receptor from *Salmonella typhimurium* as a soluble periplasmic protein has been constructed. This 18-kDa protein is exported to the periplasm, where it may be extracted by mild osmotic lysis. This isolated domain behaves as a normal, soluble protein and has been purified to homogeneity by standard techniques. The purified periplasmic domain binds aspartate with a k_D similar to that of the full-length receptor, and the binding occurs with negative cooperativity, *i.e.* the binding of one molecule of aspartate induces a conformational change that interferes with the binding of the second aspartate. Unlike the full-length receptor, the periplasmic domain undergoes a protein concentration- and aspartate-dependent monomer-dimer equilibrium. At low protein concentrations and in the absence of aspartate, the protein is monomeric. At higher protein concentrations or in the presence of saturating aspartate, the protein is dimeric. Two charge variants of the protein have been identified on native polyacrylamide gels. The more acidic form is blocked to Edman degradation, indicating that modification of the amino terminus of this protein can occur after cleavage of the signal peptide in the periplasm.

Biochemical and x-ray crystallographic studies of cell-surface receptors are difficult, because detergents are required for their solubilization. The extracellular portions (or ectodomains) of a number of receptors have been produced and characterized. The first such ectodomain to be described is that of the human insulin receptor (Johnson *et al.*, 1988). When expressed in CHO cells, the human insulin receptor ectodomain was glycosylated and processed into a heterotetrameric form similar to that of the full-length receptor. The protein was purified from the culture medium and was found to bind insulin with high affinity. Since this initial report, the characterization of many other receptor ectodomains has been reported. These include a human insulin receptor ectodomain expressed from a baculovirus vector in insect cells (Sissom and Ellis, 1989), a naturally occurring epidermal growth factor receptor ectodomain secreted by A431 cells (Basu *et al.*, 1989), and cloned ectodomains of the interleukin-1 receptor (Dower *et al.*, 1989), the lutropin/choriogonadatropin receptor (Xie *et al.*, 1990), the nerve growth factor receptor (Vissavajjhala and Ross, 1990), and the platelet-derived growth factor receptor (Duan *et al.*, 1991). All of these ectodomains retain ligand affinities very similar to those of their full-length receptor cognates. Production of the extracellular portion of the human growth hormone receptor as a periplasmic protein in *Escherichia coli* has been reported (Fuh *et al.*, 1990). This protein retains the wild type affinity for growth hormone, and the level of expression obtained in *E. coli* allowed the crystal structure of this protein to be determined (de Vos *et al.*, 1992).

Previous studies of the periplasmic domain of the aspartate receptor include both genetic and biochemical approaches. The product of the *tar* gene from *E. coli* mediates responses to both aspartate and maltose. Aspartate binds directly to the receptor (Clarke and Koshland, 1979), but maltose interacts indirectly with the receptor through the periplasmic maltose-binding protein (Hazelbauer, 1975). In 17 independently isolated aspartate receptor mutants, which affect aspartate taxis, one of three arginines (at positions 64, 69, or 72) is changed. In all of these mutants, aspartate taxis is greatly impaired, while maltose taxis is less strongly affected (Wolff and Parkinson, 1988). In a further characterization of these mutants, it was found that these mutants cause a 100- to 10,000-fold decrease in the receptor's affinity for aspartate (Mowbray and Koshland, 1990). Other studies have implicated threonine 154 in the sensing of aspartate (Lee and Imae, 1990).

Proteolytic fragments of the aspartate receptor have been purified and characterized to study the domain structure of the receptor. An endogenous protease in membrane preparations from *E. coli* cleaves the receptor at position 259 (in the cytoplasmic domain) and yields two separable fragments. One fragment corresponds to the periplasmic domain of the receptor, the two transmembrane segments, and a short segment (46 amino acids) of the cytoplasmic domain. This fragment is found in the membrane and requires detergent for solubilization from the membrane. Like the full-length receptor, this fragment is blocked to amino-terminal sequencing, and the purified protein binds aspartate with wild type affinity (Mowbray *et al.*, 1985).

In this study, the periplasmic domain of the aspartate receptor from *Salmonella typhimurium* was produced as a soluble periplasmic protein under the control of the *trc* promoter. This domain was produced by replacement of the first transmembrane segment of the receptor with the cleavable signal sequence of *E. coli* alkaline phosphatase and insertion of a stop codon before the second transmembrane segment. Induction with IPTG[1] leads to the production of an 18-kDa protein (starting with Gly-26 and ending with Arg-188) that is exported to the periplasm where it may be extracted by mild osmotic lysis. This domain of the receptor behaves as a

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[1] The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

EXHIBIT A

normal, soluble protein and has been purified to homogeneity by standard techniques. The purified protein has been biochemically characterized in an effort to learn more about the role of this domain in transmembrane signaling. The crystal structure of this protein has recently been solved (Milburn *et al.*, 1991).

EXPERIMENTAL PROCEDURES

Subcloning and Mutagenesis—The starting point for the construction of a plasmid encoding the periplasmic domain of the aspartate receptor was the plasmid pMK151, in which the first transmembrane segment of the aspartate receptor (amino acids 1–25) has been replaced with the signal sequence of *E. coli* alkaline phosphatase. In order to construct this plasmid, an *Nae*I site was inserted at position 25 in the plasmid pEMBLtar$_S$ (Falke and Koshland, 1987). An *Nae*I-*Hin*dIII fragment from this plasmid was subcloned into the plasmid pKK233-2 (Pharmacia LKB Biotechnology Inc.), which had been digested with *Nco*I, filled in using the Klenow fragment of *E. coli* DNA polymerase, and then digested with *Hin*dIII. Double-stranded oligonucleotides were then used to insert the coding region of *E. coli* alkaline phosphatase (Chang *et al.*, 1986) into the reconstructed *Nco*I site. The resulting plasmid, pMK151, produces large amounts of a receptor lacking the first transmembrane region, which is correctly targeted to the plasma membrane.

In order to produce the soluble periplasmic domain, a stop codon was inserted at position 189 in the aspartate receptor coding sequence by site-directed mutagenesis, and a fragment containing this mutation was subcloned into pMK151 to produce the plasmid pMK152. Since pMK152 does not contain the sequences necessary for production of single-stranded DNA for site-directed mutagenesis, the coding region for the periplasmic domain was subcloned into the vector pBLUESCRIPT KS+ from Stratagene. pMK152 was cut with *Bam*HI and *Sac*I, and the fragment encoding the promoter region of pKK233-2, the phoA signal sequence, and the periplasmic domain of the aspartate receptor was ligated with the pBLUESCRIPT plasmid that had been cut with the same enzymes. This vector (pMK155) was then used for construction and expression of four cysteine-containing mutants, which correspond to those made previously in the periplasmic domain of the full-length receptor (Falke and Koshland, 1987). The mutations constructed were Cys-36, Cys-106, Cys-128, and Cys-183.

Expression of the Soluble Aspartate-binding Domain—The periplasmic domain was expressed in the lacI^Q *E. coli* strain XL-1 Blue (Stratagene). Because induction of the protein with IPTG in minimal media causes growth of the cells to cease and gives little expression, rich media was used for expression. In LB medium supplemented with 200 μg/ml ampicillin and 0.04% glucose, similar induction was seen at 0.1 mM and 1 mM IPTG, and the incubation temperature (30 *versus* 37 °C) had no significant effect. For large-scale preparations, 200 liters of LB containing 200 μg/ml ampicillin and 0.04% glucose at 37 °C was inoculated with 5–6 liters of an overnight culture grown in the same medium, except that glucose was at 0.4%. When the OD_{650} had reached 0.6 (typically 2 h after inoculation), IPTG was added to 0.1 mM, and the incubation was continued for 3 additional h, at which time the OD_{650} was typically 2–3.

Purification of the Periplasmic Domain—Purification of the periplasmic domain of the aspartate receptor was accomplished by (*a*) osmotic shock and ammonium sulfate precipitation, (*b*) anion exchange chromatography, (*c*) gel filtration, (*d*) hydroxylapatite chromatography, and (*e*) fast protein liquid chromatography on Mono Q.

A) Osmotic Shock and Ammonium Sulfate Precipitation—The periplasmic domain was released from *E. coli* cells by osmotic shock (Heppel, 1971; Manoil and Beckwith, 1986) under mild conditions. This indicates that, as expected, the protein is exported into the periplasm. Cells were washed twice with wash buffer (10 mM Tris-HCl, pH 7.4, 30 mM NaCl, 0.5 mM EDTA) and then were resuspended in ice-cold spheroplast buffer (100 mM Tris-HCl, pH 8, 0.5 mM EDTA, 0.5 M sucrose, 35 μg/ml phenylmethylsulfonyl fluoride). The cells were collected by centrifugation and allowed to warm to room temperature. The cells were then quickly resuspended in 4 liters of ice-cold MilliQ water. After 2–3 min on ice, $MgCl_2$ was added to 1 mM to prevent leakage of cytoplasmic proteins from the cells. The shocked cells were removed by centrifugation, and the supernatant was saved as the shock fluid. Additions were made to bring the solution to 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM *o*-phenanthroline, 0.2 mM phenylmethylsulfonyl fluoride, after which the periplasmic domain was recovered by ammonium sulfate precipitation. In this step, 0.26 g of solid ammonium sulfate was added per ml of starting solution. After stirring in the cold for 1 h, the precipitated protein was collected by centrifugation, and the protein was resuspended in cold 10 mM Tris, pH 8.0. After an additional centrifugation step to remove insoluble material, the protein was dialyzed overnight (in Spectra/Por 3, 3500 Da cutoff dialysis tubing) against 2 liters of 10 mM Tris, pH 8.0 (exchanged at least twice with fresh buffer).

B) Anion Exchange Chromatography—The dialyzed ammonium sulfate pool was applied to a 75-ml Q-Sepharose fast flow column equilibrated with 10 mM Tris, pH 8.0. After washing with 1–2 column volumes of the starting buffer, a gradient was applied consisting of 200 ml each of 10 mM Tris, pH 8.0, and 10 mM Tris, pH 8.0, 200 mM NaCl. Fractions collected during the gradient were analyzed by SDS-PAGE. The periplasmic domain eluted at approximately 100 mM NaCl along with a yellow peak.

C) Gel Filtration Chromatography—The peak fractions from the Q-Sepharose column were pooled and concentrated by ammonium sulfate precipitation (0.3 mg of ammonium sulfate/ml of starting solution), and the pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 0.05% sodium azide. The resuspended protein was applied to a Sephadex G75 super fine column (80 cm × 1.5 cm, total volume 140 ml) equilibrated with the same buffer. Proteins were eluted with the same buffer at a flow rate of 12 ml/h. Fractions were analyzed by SDS-PAGE, and peak fractions were pooled.

D) Hydroxylapatite Chromatography—Early trials with UltraGel HA (Pharmacia) indicated that the periplasmic domain binds to this resin in Tris buffers but is eluted at a very low phosphate concentration (~1 mM). Most other proteins require a much higher phosphate concentration for elution (Gorbunoff, 1990, Gorbunoff, 1985). Thus, remaining minor impurities could be removed by adding 1 mM sodium phosphate to the pool from the G75 column and passing the solution through a small UltraGel HA column equilibrated in 50 mM Tris-HCl, pH 7.4, 1 mM NaP_i. The periplasmic domain flowed through this column, but the minor impurities were bound by the column and were removed.

E) Fast Protein Liquid Chromatography on Mono Q—After the hydroxylapatite column, the periplasmic domain was a single band on SDS-PAGE gels, but two bands were seen on native gels. In order to separate these forms, a final Mono Q column was run. The hydroxylapatite pool was diluted at least 3-fold with cold water, passed through a 0.2 microfilter, and applied to a Mono Q HR 10/10 column equilibrated in 10 mM Tris, pH 8.0. After washing with the starting buffer, the protein was eluted with a gradient of 10 mM Tris, pH 8.0, and 10 mM Tris, pH 8.0, 200 mM NaCl over 60 min at a flow rate of 3 ml/min. Two peaks of protein were seen, and when fractions were analyzed on native gels, the later eluting peak was seen to migrate faster on the gels. The larger, early peak of protein is the form that has been used for the crystallization and structure determination. For long-time storage, sodium azide was added to 0.05%. In this form, the protein appears stable at 4 °C.

Biochemical Characterization of the Periplasmic Domain—Determination of the stoichiometry of aspartate binding requires an accurate estimation of the protein concentration in the binding assays. Calculation of an extinction coefficient at 280 nm from the amino acid composition of the periplasmic domain (Scopes, 1987) yields a value of 0.8 for a 1 mg/ml protein solution. As an independent check on this estimate, the purified Cys-183 periplasmic domain protein was used to correlate the number of cysteine residues with the absorbance of the protein at 280 nm. In one procedure, the reduced Cys-183 periplasmic domain was treated with an excess of [^{14}C] iodoacetamide, and the amount of incorporated radioactivity was compared with the absorbance at 280 nm. This yielded an extinction coefficient of 1.0 for a 1 mg/ml solution. In a second procedure, the Ellman reaction (Riddles *et al.*, 1983) was used to assay the cysteine content of the purified protein. The extinction coefficient calculated by this method was 0.87 for a 1 mg/ml solution. Based on a combination of these procedures, an extinction coefficient of 0.9 mg^{-1} ml at 280 nm has been used. This estimate has recently been confirmed by amino acid analysis.[2]

Aspartate binding was measured essentially as described (Clarke and Koshland, 1979) with the exception that Millipore Ultrafree-MC 10,000 dalton nominal molecular weight cutoff filter units were used to separate bound and free aspartate. Briefly, the periplasmic domain was incubated on ice with varying amounts of [^{14}C]aspartate or [^{3}H] aspartate in a total volume of 200 μl. After 5 min, 95-μl aliquots were

[2] H. P. Biemann and D. E. Koshland, unpublished results.

added to tubes containing either 1 μl of a 50 mM aspartate solution or 1 μl of H_2O. In each case, free aspartate was separated from that bound by the periplasmic domain, and the amount of aspartate remaining in solution was determined by counting 10–15-μl aliquots in 4 ml of scintillation fluid. It was important to add 100–200 μl of water to the scintillation fluid (Packard Scint-A XF) to avoid precipitation of the aspartate and reduction in counting efficiency, especially in samples containing an excess of cold aspartate. The amount of free aspartate was calculated from the concentration of aspartate remaining in solution in the $+H_2O$ tubes, and the amount of aspartate specifically bound was calculated as the difference between the +cold aspartate and $+H_2O$ tubes. The data were analyzed by Scatchard analysis (Scatchard, 1949) or by nonlinear curve fitting to the Hill equation (Hill, 1913) using the program KaleidaGraph 2.1.

Sedimentation equilibrium experiments were performed in the Beckman TL 100 Preparative Ultracentrifuge as previously described for the full-length aspartate receptor (Milligan and Koshland, 1988). The Cys-183 periplasmic domain mutant, radioactively labeled with ^3H or ^{14}C iodoacetamide, was used in these experiments. The buffer contained 50 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mg/ml dextran T40, 1 mg/ml bovine serum albumin, and 0.05% sodium azide. The partial specific volume of the periplasmic domain calculated from the amino acid composition (McMeekin and Marshall, 1952) is 0.73 ml/g.

Gel electrophoresis was performed using the BioRad MiniProtean apparatus, using the standard recipes of Laemmli (Laemmli, 1970) for SDS-PAGE. Native gels were run in the same apparatus and using the same buffers, prepared without SDS. Native gels were run at 100 V at 4 °C. For many applications (including the analysis of column fractions during purification), the Pharmacia PhastGel system was used in either the SDS-PAGE or the native gel mode.

Analytical gel filtration was performed at room temperature using a Waters model 510 high performance liquid chromatography system and a BioRad TSK-250 (600 × 7.5 mm) gel filtration column. The column was run at 1 ml/min in 100 mM NaP_i, and aspartate was added to a concentration of 1 mM where indicated. Proteins eluting from the column were detected by measuring absorption changes at 215 or 280 nm.

Fluorescence measurements were made on an SLM-Aminco 4800 S spectrofluorometer. The protein concentration was approximately 1 μM in a buffer consisting of 50 mM Tris-HCl, pH 7.4. Aspartate was added to a final concentration of 1 mM from a 1 M stock. All measurements were made at 20 °C. Excitation was at 295 nm, and the emission spectrum was scanned between 300 and 400 nm.

RESULTS

The periplasmic domain of the aspartate receptor of *S. typhimurium* has been produced as a soluble periplasmic protein in *E. coli*. We first constructed a plasmid that expresses an aspartate receptor in which the first transmembrane segment had been replaced with the signal sequence of *E. coli* alkaline phosphatase. This protein is directed to the membrane and binds aspartate but does not mediate chemotaxis to aspartate (Fig. 3). Insertion of a stop codon before the second transmembrane segment leads to the production of a soluble protein in the periplasm corresponding to the aspartate-binding domain of the receptor.[3]

Expression and Purification of the Periplasmic Domain—The *E. coli* strain XL-1 Blue harboring the plasmid pMK152, which expresses the periplasmic domain of the *S. typhimurium* aspartate receptor, was grown in a 200-liter fermenter with a yield of 500–800 g of cells. In a typical preparation starting with 600 g of cells, a final yield of 250 mg of pure periplasmic domain was obtained. The osmotic shock procedure provides a substantial purification, because all of the cytoplasmic and membrane proteins are discarded in this step. After the ion-exchange and gel filtration columns, the protein is greater than 95% pure, and the final hydroxylapatite column removes the remaining impurities. A final MonoQ column separates two charge variants of the protein (see below). An SDS-polyacrylamide gel summarizing a typical purification procedure is shown in Fig. 1.

Expression and Purification of Cysteine-containing Periplasmic Domain Proteins—Because a cysteine at position 36 does not interfere with signaling by the full-length receptor (even in the disulfide form) (Falke and Koshland, 1987), a mutant periplasmic domain containing this cysteine was expressed and purified for crystallographic purposes. The wild type periplasmic domain did not form diffraction quality crystals, but crystals of the cross-linked Cys-36 mutant diffract to at least 1.9 Å (Milburn *et al.*, 1991). Purification of the cysteine-containing mutants was carried out essentially as described for the wild type fragment. In early purification trials, 5 mM dithiothreitol (DTT) was added to the buffers in an attempt to prevent disulfide formation in the Cys-36 mutant. Subsequently, it was found that the protein was already cross-linked (either *in vivo* or during the osmotic shock) and that this concentration of DTT was unable to reduce the cross-linked Cys-36 periplasmic domain. Very high concentrations of DTT (>50 mM) were required to reduce this disulfide bond. Conversely, the Cys-183 periplasmic domain was found in the reduced form when 5 mM DTT was included in the buffers. Oxidative catalysts, such as $Cu(phenanthroline)_3$ were required to promote disulfide formation at this position.

Aspartate Binding to the Periplasmic Domain—Aspartate binding by the periplasmic domain protein was measured to determine the role of the transmembrane segments of the receptor in ligand binding. The purified periplasmic domain binds aspartate with high affinity, indicating that the transmembrane segments of the receptor are not required for aspartate binding. However, binding of aspartate by the periplasmic domain is not identical to binding by the full-length receptor. As shown in Fig. 2, simple Michaelian binding is not observed, and the binding curves do not reach a saturating value in these experiments. Furthermore, as shown in Fig. 3, Scatchard analysis of the binding yields a distinctly nonlinear plot. Concave upward Scatchard plots indicate negative cooperativity or a mixture of sites with different affinities. The latter seems unlikely, since the x-ray crystal structure shows that the apoprotein is symmetrical about the crystallographic axis between the two subunits of the dimer (Milburn *et al.*, 1991). A nonlinear least-squares fit of the data to the Hill (Hill, 1913) equation follows.

$$N = L_{max}(L)^n/((L)^n + (K_{0.5})) \quad \text{(Eq. 1)}$$

N is the concentration of aspartate bound and L is the concentration of free aspartate, which gives a Hill coefficient

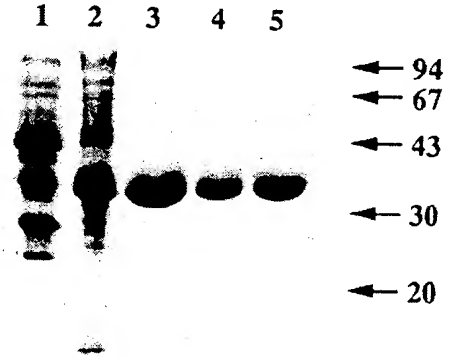

FIG. 1. **SDS-polyacrylamide gel showing the progress of purification of the periplasmic domain protein.** Pooled fractions were run on a 15% SDS-PAGE gel. *Lane 1*, osmotic shock fluid; *lane 2*, ammonium sulfate pool; *lane 3*, Q-Sepharose pool; *lane 4*, G75 pool; *lane 5*, hydroxylapatite pool.

[3] D. L. Milligan and D. E. Koshland, unpublished observations.

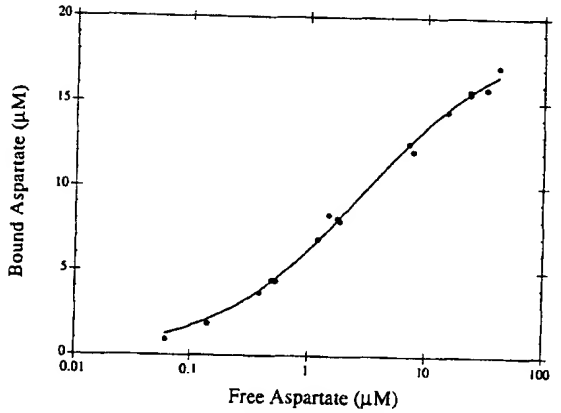

FIG. 2. **Aspartate binding to the periplasmic domain of the aspartate receptor.** Binding was measured as described under "Experimental Procedures." The curve is a nonlinear least-squares fit to the Hill equation, $n = N_{max}(L)^n/((L)^n + (K_{0.5}))$, where N is the bound aspartate, N_{max} is the maximum number of binding sites, L is the concentration of free aspartate, and n is the Hill coefficient. The protein subunit concentration in the assay was 17 μM.

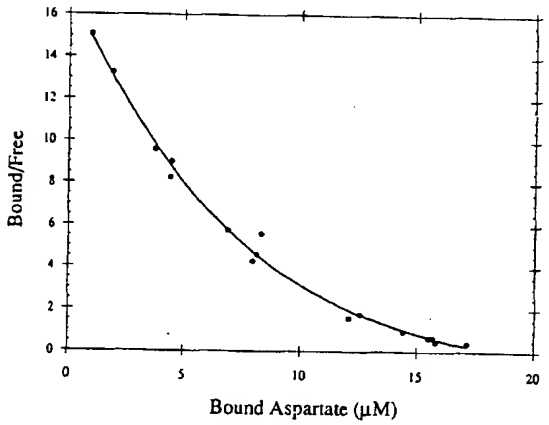

FIG. 3. **Scatchard analysis of aspartate binding.** The binding data from Fig. 2 are plotted on a Scatchard graph to illustrate the nonlinearity of the curve. Concave-up Scatchard plots indicate negative cooperativity or a mixture of sites with different affinities.

(n) of 0.7. Like the concave-up Scatchard plots, a Hill coefficient less than one indicates negative cooperativity or a mixture of sites with different affinities (Levitzky, 1984). The $K_{0.5}$ calculated from a computer fit of the data to Equation 1 is 2 μM, consistent with the affinity of the full-length receptor for aspartate, which is in the range of 1–5 μM (Clarke and Koshland, 1979; Foster *et al.*, 1985). At a protein concentration of 17 μM, the calculated concentration of binding sites was 18–19 μM, consistent with one binding site/subunit.

Analytical Gel Filtration—The full-length aspartate receptor is a dimer of 60-kDa subunits in the presence and absence of aspartate. However, the receptor elutes as a 240-kDa protein rather than the expected 120 kDa on gel filtration columns, presumably due to an asymmetric structure of the detergent-solubilized dimer (Milligan and Koshland, 1988). The oligomeric structure of the periplasmic domain has been studied by performing analogous gel filtration experiments on the purified wild type and cross-linked Cys-36 proteins in the presence and absence of aspartate. As shown in Fig. 4, the apparent size of the periplasmic domain changes in the presence and absence of saturating aspartate. The apparent molecular weight of the periplasmic domain in the absence of aspartate is 18.5 kDa, while in the presence of aspartate it is

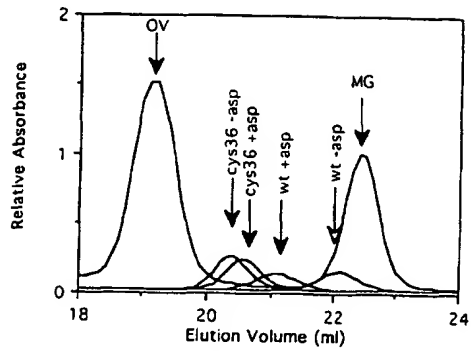

FIG. 4. **Gel filtration of the wild type and Cys-36 periplasmic domain proteins in the presence and absence of aspartate.** Gel filtration experiments were performed on a BioRad TSK-250 column equilibrated with 100 mM NaP_i, pH 7, and, where indicated, aspartate was added to a final concentration of 1 mM in the sample and the column buffer. Each of the curves represents a separate run. The positions of marker proteins, ovalbumin (*OV*), 44 kDa, and myoglobin (*MG*), 17 kDa, are indicated by the *arrows*, as are the positions of the wild type and cross-linked periplasmic domain proteins.

25 kDa. The cross-linked Cys-36 periplasmic domain elutes as a 30.5-kDa protein, and aspartate causes a slight decrease in the apparent size (to 29 kDa).

Analysis of Oligomeric Structure by Sedimentation Equilibrium—The gel filtration studies suggest that the periplasmic domain undergoes an aspartate-dependent dimerization reaction. In order to test this hypothesis, sedimentation equilibrium experiments were conducted in a tabletop ultracentrifuge, as described previously for the full-length receptor (Milligan and Koshland, 1988). These experiments were conducted using [^{14}C]iodoacetamide or [^{3}H]iodoacetamide to label the Cys-183 mutant periplasmic domain. As expected from earlier experiments with full-length receptor mutants (Falke and Koshland, 1987), the cysteine at this position is much more easily reduced and modified than Cys-36. Among the advantages of sedimentation equilibrium methods are the lack of influence of protein shape on the results and the ability to use radiolabeled protein to analyze sedimentation at very low protein concentrations.

Sedimentation equilibrium experiments confirmed the earlier gel filtration experiments and showed that not only the aspartate but also the protein concentration affects the oligomeric structure of the periplasmic domain of the aspartate receptor. Fig. 5 shows the raw sedimentation equilibrium data in the presence and absence of saturating aspartate at the different protein concentrations indicated. At low protein concentrations, the sedimentation profile was considerably steeper in the presence of aspartate than in its absence, while at higher protein concentrations, there was no difference between the plus and minus aspartate curves. These results are summarized in Fig. 6, in which the apparent molecular weight (M) was calculated from the following equation.

$$M(1 - v\rho) = 2RT(\mathrm{d} \ln c/\mathrm{d}r^2)/w^2 \qquad \text{(Eq. 2)}$$

ρ is the solution density, R is the gas constant, T is the absolute temperature, c is the protein concentration, r is the radial distance from the center of rotation, and w is the angular velocity in rad/sec). By ignoring the nonlinearity of the curves, an average molecular weight could be calculated from the linear part of the curve at different starting protein concentrations. This analysis indicates that a shift toward higher molecular weight occurs as the protein concentration is increased. In the absence of aspartate the calculated molecular mass varies from 20 kDa at low protein concentrations

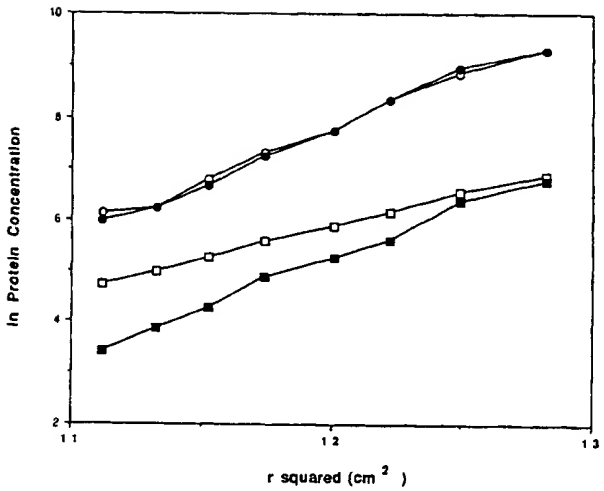

FIG. 5. **Sedimentation equilibrium of the aspartate receptor periplasmic domain.** Sedimentation equilibrium studies were conducted in the Beckman TL100 tabletop Ultracentrifuge, as described under "Experimental Procedures." In this graph, the natural log of the protein concentration is plotted against the r^2, where r is the radial position during centrifugation at two different starting protein concentrations and in the absence (*open symbols*) or presence (*closed symbols*) of (1 mM) aspartate. The protein concentration was determined by fractionation of the contents of each tube after centrifugation for at least 48 h. The starting protein concentrations were 0.014 mg/ml (□, ■) or 4.7 mg/ml (○, ●).

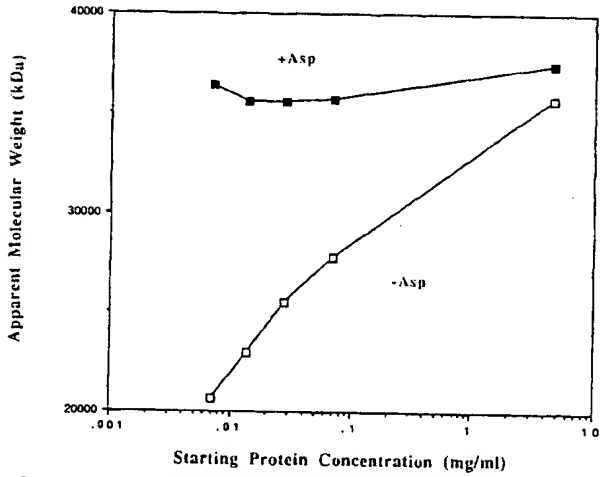

FIG. 6. **Dependence of the molecular weight of the periplasmic domain on protein concentration in the presence and absence of aspartate.** The apparent molecular weight of the periplasmic domain calculated from the type of curves shown in Fig. 5 is plotted against the starting protein concentration on a semilog plot. Parallel experiments were conducted in the absence of aspartate (□) and in the presence of 1 mM aspartate (■).

to 35 kDa at high concentrations. In the presence of aspartate, the calculated molecular weight is constant over the range of protein concentrations tested. Since the subunit molecular mass of the periplasmic domain is 18 kDa, these data indicate that the protein undergoes an aspartate- and protein concentration-dependent monomer-dimer equilibrium. The apparent dissociation constant for the monomer-dimer equilibrium in the absence of aspartate is in the range of 0.01–0.1 mg/ml (0.5–5 μM) while that in the presence of saturating aspartate is at least two orders of magnitude lower.

In order to measure the dependence of the monomer-dimer equilibrium on the concentration of aspartate, sedimentation equilibrium measurements were performed at varying concentrations of aspartate. As shown in Fig. 7, increasing aspartate causes a shift to a higher apparent molecular weight, indicating that aspartate binding shifts the monomer-dimer equilibrium toward the dimeric form. At saturating aspartate concentration, the apparent molecular weights do not depend on protein concentrations, consistent with the results discussed above. The shift in apparent molecular weight occurs over a range of 0.1–10 μM aspartate, with the midpoint occurring at approximately 1 μM aspartate, consistent with the measured affinity of the receptor for aspartate.

Fluorescence Measurements—The periplasmic domain has a single tryptophan at position 57, close in the primary sequence to 3 arginine residues at positions 64, 69, and 73, which have been implicated in the binding of aspartate (Milburn *et al.*, 1991, Mowbray and Koshland, 1990, Wolff and Parkinson, 1988). The sensitivity of this tryptophan residue to ligand binding was tested by measuring the effect of aspartate on the fluorescence spectrum of the periplasmic domain protein. Fig. 8 shows a fluorescence emission spectrum between 300 and 400 nm when excitation is at 295 to preferentially excite tryptophan. As shown in this figure, the intensity

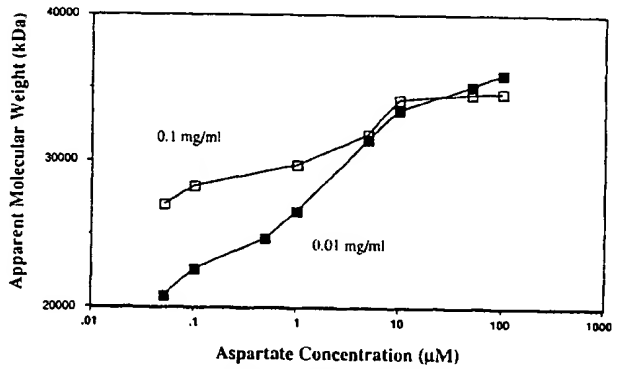

FIG. 7. **Effect of aspartate concentration on sedimentation equilibrium of the periplasmic domain.** Sedimentation equilibrium experiments were conducted at two different starting protein concentrations, 0.01 mg/ml (■) and 0.1 mg/ml (□) over a range of aspartate concentrations from 0.05 to 100 μM. The apparent molecular weight of the periplasmic domain protein is plotted against the aspartate concentration on a semilog graph.

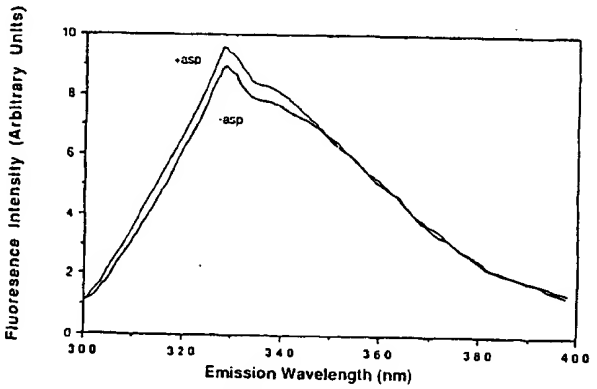

FIG. 8. **Fluorescence emission spectra in the presence and absence of aspartate (*asp*).** Fluorescence emission was measured from 300 to 400 nm, with excitation at 295 to preferentially excite tryptophan residues. The excitation slit was set at 2 nm, and the emission slit was 4 nm. Where indicated, aspartate was added to a final concentration of 1 mM from a 1 M stock solution. In a separate experiment, addition of an equal volume (3 μl) of water had no effect on the emission spectrum.

of fluorescence emission increases (~8% between 320 and 340 nm) when aspartate is added to saturation. Such an increase in tryptophan fluorescence is usually associated with a shift to a more hydrophobic environment (Cantor and Schimmel, 1980). The sensitivity of this tryptophan to aspartate binding provides another avenue to the study of conformational changes that mediate transmembrane signaling.

Native Gel Electrophoresis—Initial crystallization trials of the periplasmic domain of the aspartate receptor were conducted with material that migrated as a single band on SDS-PAGE. This material did not produce diffraction quality crystals (Jancarik *et al.*, 1991). Subsequently, it was found that this apparently pure material, which migrates as a single band on SDS-PAGE, migrates as two bands in a native polyacrylamide gel. The more slowly migrating (more basic) form is present at three to five times the amount of the faster migrating (more acidic) form. These two forms appear to result from covalent modification of one of the forms, because neither the presence of 4 M urea nor boiling of the protein affected the ratio of the two forms on native gels. The two forms could be separated on a MonoQ column, from which the more acidic form eluted at higher salt concentrations. The difference between the two forms was found to be present at the amino terminus of the more acidic form. Edman sequencing of the more basic form yielded overlapping sequences beginning with the predicted methionine residue following the cleavage site, Gly-26 or Gly-27 (numbering of the full-length receptor). The lack of sequence information from the more acidic form indicated that the amino terminus of this form is modified.

DISCUSSION

The periplasmic domain of the aspartate receptor has been purified and biochemically characterized in an effort to learn more about the role of this domain in transmembrane signaling. While the full-length receptor is continually dimeric in the presence and absence of aspartate, the periplasmic domain of the receptor undergoes an aspartate- and protein concentration-dependent monomer-dimer equilibrium. The protein binds aspartate and undergoes a conformational change upon binding, as shown by an increase in the fluorescence intensity of the single tryptophan residue in this protein. Cysteine mutants, corresponding to mutations made previously in the periplasmic domain of the full-length receptor, have been constructed and used as sites for radioactive labeling and for determination of the stoichiometry of aspartate binding. In addition, use of the cross-linked Cys-36 periplasmic domain has been instrumental in the solution of the crystal structure of this domain (Jancarik *et al.*, 1991, Milburn *et al.*, 1991). Expression of the periplasmic domain of the aspartate receptor as a fusion to the alkaline phosphatase (phoA) signal sequence simplified purification of the protein. The signal sequence was cleaved during transport to the periplasm, and the protein could be released from the periplasm by mild osmotic shock. The protein could be purified in 100-mg amounts in less than 1 week.

Binding of aspartate to the periplasmic domain indicates that neither of the transmembrane segments of the receptor is required for formation of the ligand binding site and demonstrates that this domain of the receptor is able to fold independently into a functional conformation. The stoichiometry of binding is one aspartate/subunit. The affinity of the periplasmic domain for aspartate is very similar to that of the full-length receptor, but, unlike the full-length receptor (Clarke and Koshland, 1979, Foster *et al.*, 1985), the periplasmic domain does not exhibit simple Michaelian binding. Scatchard plots of binding to the periplasmic domain are concave-up, and a nonlinear least squares fit to the Hill equation yield a Hill coefficient that is less than one. Since the two binding sites in the dimeric protein are identical (Milburn *et al.*, 1991), aspartate must bind to the periplasmic domain with negative cooperativity. Aspartate causes a shift from monomer to dimer, and the crystal structure shows that aspartate binds at the dimer interface (Milburn *et al.*, 1991). Hence, it is clear that binding of the first aspartate must induce a conformational change that greatly lowers the affinity at the second site.

Unlike the full-length aspartate receptor, which is dimeric in the presence and absence of aspartate, the periplasmic domain of the receptor undergoes a monomer-dimer equilibrium that is both protein concentration- and aspartate-dependent. This result suggests that the transmembrane segments and/or the cytoplasmic domain of the receptor take part in inter-subunit contacts, which lead to the higher stability of the full-length receptor dimer. Alternatively, the presence of the transmembrane or cytoplasmic domains could affect the conformation of the periplasmic portion of the full-length receptor such that the subunit affinity is higher.

Sedimentation equilibrium experiments were conducted to further study the protein concentration dependence and the aspartate dependence of the oligomeric structure of the periplasmic domain. These experiments clearly showed the dependence of the oligomeric structure on protein concentration and aspartate. At protein concentrations below 0.1 mg/ml (5 μM), the periplasmic domain was predominantly monomeric in the absence of aspartate. At 5 mg/ml (250 μM), the protein was dimeric. However, addition of saturating aspartate at any of the protein concentrations tested lead to dimerization of the periplasmic domain, showing that binding of aspartate caused a considerable increase in the affinity of periplasmic domain subunits for each other. These experiments suggest that aspartate binding increases the subunit affinity at least 100-fold. At low and intermediate protein concentrations, dimerization occurred over a range of 1–10 μM aspartate, consistent with the measured affinity of the protein for aspartate. The high effective concentration of the full-length receptor in the membrane, coupled with the fact that the receptor has a higher subunit affinity than the isolated periplasmic domain, clearly indicates that the full-length receptor is always present as a dimer in the membrane.

After purification of the periplasmic domain to apparent homogeneity on SDS-PAGE gels, the protein migrates as two bands on native gels. It was subsequently found that there is a covalent modification of 15–20% of the periplasmic domain protein, which causes the loss of the positive charge at the amino terminus and prevents Edman degradation. Both forms bind aspartate with equal affinity, and no other differences between the protein have been observed. Modification of the amino terminus of the periplasmic domain must occur after removal of the phoA signal sequence by leader peptidase. Since the active site of leader peptidase is located on the outer surface of the inner membrane (Wolfe *et al.*, 1983), modification of the periplasmic domain must occur in the periplasm of *E. coli*. Determination of the exact nature of this modification may uncover previously unknown enzymatic reactions that occur in the periplasm.

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